Jundishapur J Microbiol. 2020 August; 13(8):e101767. doi: 10.5812/jjm.101767.
Published online 2020 November 21. **Research Article**

Antifungal Susceptibility of Non-*albicans Candida* Species in A Tertiary Care Hospital, Bulgaria

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Received 2020 February 12; **Revised** 2020 September 28; **Accepted** 2020 September 30.

Abstract

Background: Emerging non-*albicans Candida* (NAC) species are a major threat because of their intrinsic or acquired resistance to routinely applied antifungal agents.
Objectives: The purpose of our study was to reveal *in vitro* activity of nine antifungal agents against NAC isolates.
Methods: A total of 67 NAC (27 *Candida glabrata*, 10 *C. tropicalis*, 6 *C. krusei*, 6 *C. parapsilosis*, 4 *C. lusitaniae*, 4 *C. lipolytica*, etc.) were identified and tested. The antifungal susceptibility was estimated on the basis of minimum inhibitory concentrations (MIC).
Results: Overall, 13 species were determined, of which *C. glabrata* was the most common (40.3%), followed by *C. tropicalis* (14.9%), *C. krusei*, and *C. parapsilosis* (8.9 % each). Forty-nine NAC isolates (73.13%) demonstrated decreased susceptibility to one or more antifungals, and 18 of them were resistant to all azoles. Out of 27 *C. glabrata*, 12 (44.4%) were resistant to fluconazole with MICs: 32 ->128 μg/mL and 15 (55.6%) were intermediate with MICs: 8 - 16 μg/mL Non-*albicans Candida* revealed a good susceptibility to echinocandins. Amphotericin B resistance was found in 5.97% of the isolates. Of particular interest was the detection of 6 (8.95%) multidrug-resistant NAC, which expressed resistance to azoles and echinocandins and/or amphotericin B.
Conclusions: About one-fourth of the studied NAC were resistant to all azoles. These findings as well as the detection of several multidrug-resistant isolates determine the necessity of susceptibility testing of clinically important yeast isolates and control of the antifungal drugs in our hospital.

Keywords: Non-*albicans Candida*, Resistance, Antifungal Agents

1. Background

Over the past decades, the incidence of *Candida* infections among hospitalized patients has progressively increased. This is a consequence of the frequent usage of broad-spectrum antibiotics, admission to critical care settings, invasive manipulations, surgical procedures, indwelling devices, transplantation, chemotherapy, immunosuppression, etc. (1-6). In the USA, the rate of fungal infections rose from 6% in 1980 to 10.4% in 1990 and about 80% of them were caused by *Candida* species (7) Also, the proportion of *Candida* spp. among isolates from blood cultures increased from 8% in 1995 to 12% in 2002 (8).

Nowadays, a shift toward non-*albicans Candida* (NAC) species has been observed (9). Emerging NAC such as *Candida glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* can cause various superficial, disseminated and deep-tissue infections, but the species involvement depends on infection sites and geography regions (10, 11). As is well known, NACs have reduced sensitivity to clinically relevant antifungal agents due to intrinsic or acquired resistance (12, 13). *Candida krusei* is considered inherently resistant to fluconazole and *C. glabrata* may be echinocandin resistant in association with cross-resistant to azoles (14). With the increasing rate of *Candida* infections and the emergence of resistant strains, it is essential to perform routine antifungal susceptibility testing of clinical yeast isolates (15). Despite a large number of reports on spreading *Candida* species worldwide and detection of yeasts resistant to antifungals, there are scant data for Bulgaria (16, 17).

2. Objectives

The purpose of our study was to reveal *in vitro* activity of nine antifungal agents against NAC isolates.

3. Methods

3.1. Yeast Isolates

The object of this study was 67 non-repeated NAC isolates obtained from clinical specimens of 67 patients treated in University Hospital, Dr. G. Stranski"-Pleven, Bulgaria from September 2016 to December 2017. NACs were isolated from urines (31), throat swabs and oral cavity samples (9), wound aspirates (7), lower respiratory tract samples (6), blood cultures (4), vaginal swabs (3), feces (3), ear swabs (3), and central venous catheter (1). Patients' samples were collected from the intensive care unit (28), urology (9), surgery (8), hematology (7), oncology (7), nephrology (4), and pediatrics (4).

3.2. Species Identification

The preliminary identification of isolates was based on colonies coloration on CHROM agar *Candida* (BD, UK) and microscopic characteristics on cornmeal agar. The final identification was made by MIKROLATEST *CANDIDA* test 21 (Erba Lachema, CZ) and Vitek 2 compact system (Bio Merieux, France).

3.3. Antifungal Susceptibility Testing

Micronaut-AM (Merlin Diagnostika GmbH, Germany) was used to determine the minimum inhibitory concentrations (MICs) of amphotericin B (APH), 5-fluorocytosine (FCY), fluconazole (FCA), voriconazole (VOR), posaconazole (POS), itraconazole (ITR), micafungin (MIF), anidulafungin (ANF), and caspofungin (CAS). The concentration ranges of drugs used in each row was the following: APH (0.031 - 16 μg/mL), FCY (0.0625 - 32 μg/mL), FCA (0.002 - 128 μg/mL), VOR and POS (0.0078 - 8 μg/mL), ITR (0.031 - 4 μg/mL), MIF, ANF and CAS (0.002 - 8 μg/mL). Depending on color change (pink) of the growth control, the plates were incubated 22 - 48 hours at 35°C before reading. The MICs were interpreted in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2018 (18), with MIC breakpoints in μg/mL. For *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*: APH susceptibility (S) $\leq$ 1 and resistance (R) >1. For *C. glabrata*: FCA S $\leq$ 0,002 and R > 32. For *C. tropicalis* and *C. parapsilosis*: FCA S $\leq$ 2 and R > 4; ITR S $\leq$ 0.125 and R > 0.125; POS S $\leq$ 0.064 and R > 0.064; VOR S $\leq$ 0.125 and R > 0.25. For *C. glabrata*, *C. krusei*, and *C. tropicalis*: ANF S $\leq$ 0.064 and R > 0.064. For *C. glabrata*: MIF S $\leq$ 0.032 and R > 0.032. For *C. parapsilosis*: ANF S $\leq$ 0.002 and R > 4; MIF S $\leq$ 0.002 and R > 2. The data for FCY were evaluatd as per recommendations of the manufacturer (Micronaut-AM, Merlin Diagnostika GmbH, Germany): S $\leq$ 4 μg/mL, intermediate (I) 8 - 32 μg/mL and R > 32 μg/mL.

3.4. Multidrug Resistance

The simultaneous resistance to $\geq$1 drug in $\geq$ 2 classes of antifungals was accepted as multidrug resistance (19).

4. Results

Out of 67 non-*albicans Candida* isolates obtained from clinical specimens, 27 (40.3%) were identified as *C. glabrata*, 10 (14.9%) -as *C. tropicalis*, 6 (8.9%)- as *C. krusei* and 6 (8.9%) - as *C. parapsilosis*. The other species comprised overall 27% as follows: *C. lusitaniae* (4), *C. lipolytica* (4), *C. guilliermondii* (2), *C. utilis* (2), *C. famata* (2) and *C. norvegensis*, *C. lambica*, *C. pelliculosa*, *C. kefyr* (each one 1). From 27 *C. glabrata* isolates, 20 (74.1%) were recovered from urines. The results from susceptibility testing are given in Table 1 as MIC ranges, MIC_{50} and MIC_{90}, as well as in Tables 2 and 3 as MIC ranges. Susceptible to nine antifungal agents were 18 yeasts (26.87%): *C. parapsilosis* (6), *C. tropicalis* (4), *C. lusitaniae* (4), *C. utilis* (2), *C. pelliculosa* (1), and *C. kefir* (1). The remaining 49 (73.13%) demonstrated decreased susceptibility to one or more antifungals.

Table 1. Susceptibility of *Candida glabrata* to Antifungal Agents: MIC Ranges, MIC_{50} and MIC_{90}

Antifungal Agents	*Candida glabrata* (n = 27)		
	MIC Ranges (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)
Fluconazole	8 - >128	16	> 128
Itraconazole	4 - >4	4	4
Posaconazole	0.25 - > 8	1	4
Voriconazole	0.0625 - > 8	0.25	8
Anidulafungin	0.015 - 0.0625	0.031	0.0625
Caspofungin	0.0625 - 0.25	0.125	0.25
Micafungin	0.015 - 0.031	0.015	0.015
5-fluorocytosine	< 0.0625 - 4	0.0625	0.125
Amphotericin B	0.5 - 2	1	1

Abbreviation: MIC, minimum inhibitory concentrations.

High level resistance to all azoles was observed in overall 18 yeasts (26.87%): *C. glabrata* (12), *C. tropicalis* (3), *C. famata* (2), and *C. lipolitica* (1). Four yeasts (5.97%) were resistant to amphotericin B with MICs of 2 μg/mL. As shown in Table 1*C. glabrata* showed decreased susceptibility to azoles with fluconazole MIC_{90} > 128 μg/mL. Of overall 27 isolates, 12 (44.4%) were resistant to fluconazole (MICs: 32 - > 128 μg/mL) and 15 (55.6%) were intermediate susceptible (MICs: 8 - 16 μg/ml). The MICs of itraconazole, posaconazole and voriconazole were elevated (MIC_{90s} of 4 - 8 μg/mL).

Table 2. Minimum Inhibitory Concentrations Ranges to Antifungal Agents of the Less Common Non-*albicans Candida* Species

Candida spp.	Antifungal Agents (MIC Ranges in μg/mL)								
	FCA	ITR	POS	VOR	ANF	CAS	MIF	FCY	APH
C. tropicalis (n = 10)	2- > 128	0.125- > 4	0.0625- > 8	0.125 - > 8	0.015 - > 8	0.125 - 4	0.031- > 8	< 0.0625 - 32	1 - 2
C. krusei (n = 6)	32 - 64	1 - 4	0.0625-0.125	0.125	0.031 - 0.0625	0.25	0.031 - 0.125	1 - 8	1
C. parapsilosis (n = 6)	0.5 - 2	< 0.031 - 0.125	< 0.0078-0.015	< 0.0078 - 0.0625	< 0.002 - 0.031	0.125	<0.015 - 0.0625	0.0625 - 0.125	0.5 - 1
C. lusitaniae (n = 4)	0.5	< 0.031	<0.0078	< 0.0078	< 0.002 - 0.0625	0.0625 - 0.125	0.015 - 0.031	0.5 - 2	0.5
C. lipolitica (n = 4)	16 - 32	0.031 - 4	0.031 - 1	0.031 - 1	0.015 - 1	0.125 - 8	0.015 - > 8	0.0625 - 0.5	0.5 - 1

Abbreviation: MIC, minimum inhibitory concentrations; MIC, minimum inhibitory concentrations; FCA, fluconazole; ITR, itraconazole; POS, posaconazole; VOR, voriconazole; ANF, anidulafungin; CAS, caspofungin; MIF, micafungin; FCY, 5-fluorocytosine; APH, amphotericin B.

Table 3. Minimum Inhibitory Concentrations Ranges to Antifungal Agents of the Rare Non-*albicans Candida* Species

Candida spp.	Antifungal Agents (MIC Ranges in μg/mL)								
	FCA	ITR	POS	VOR	ANF	CAS	MIF	FCY	APH
C. guilliermondii (n = 2)	2 - 8	0.5 - 4	0.125	0.125	0.015 - 0.0625	0.25	0.0625	< 0.0625	0.5
C. utilis (n = 2)	0.5 - 2	< 0.031 - 0.5	< 0.007-0.125	< 0.0078 - 0.0625	0.002 - 0.015	0.125	0.015 - 0.0625	< 0.0625 - 4	0.5 - 1
C. famata (n = 2)	> 128	> 4	8	8	0.031	0.125	0.015	< 0.0625	1 - 2
C. norvegensis (n = 1)	128	4	0.125	0.25	0.0625	0.0625	0.125	4	1
C. lambica (n = 1)	16	< 0.031	0.031	0.031	0.125	0.125	0.015	2	0.5
C. pelliculosa (n = 1)	0.5	0.0625	0.0625	0.031	0.015	0.0625	0.015	2	0.5
C. kefyr (n = 1)	0.25	< 0.031	< 0.0078	< 0.0078	0.031	0.0625	0.0625	< 0.0625	1

Abbreviation: MIC, minimum inhibitory concentrations; MIC, minimum inhibitory concentrations; FCA, fluconazole; ITR, itraconazole; POS, posaconazole; VOR, voriconazole; ANF, anidulafungin; CAS, caspofungin; MIF, micafungin; FCY, 5-fluorocytosine; APH, amphotericin B.

C. glabrata revealed good susceptibility to echinocandins (MIC_{90} of anidulafungin - 0.0625 and MIC_{90} of micafungin - 0.015 μg/mL), 5-fluorocytosine (MIC_{90} - 0.125), and amphotericin B (MIC_{90} - 1 μg/mL).

The MICs of echinocandins for the other NAC are shown in Tables 2 and 3. Only 2 isolates (1 *C. tropicalis* and 1 *C. lipolitica*) indicated high MICs to anidulafungin (MICs: > 8 and 1 μg/mL, respectively) and micafungin (MICs > 8 μg/mL). Six (8.95%) of NAC were multidrug resistant, including *C. glabrata* (1), *C. tropicalis* (3), *C. lipolitica* (1), and *C. famata* (1). These isolates showed high-level resistance to all azoles with a combination of echinocandin and/or amphotericin B resistance (Table 4).

5. Discussion

Antifungal susceptibility of clinical non-*albicans Candida* isolates was presented in this study. Overall, 13 species were found of which *C. glabrata* was the most common, followed by *C. tropicalis*, *C. krusei*, and *C. parapsilosis*. These four species make up about 70% of the tested isolates. Many authors reported predominance of the same NAC in hospitalized patients (8-10, 17, 20-23), but the rate of a definitive species varied according to the study designs. Basseti et al. (24) detected *C. parapsilosis* and *C. glabrata* as the second and third species isolated from blood cultures in the period 2008-2010.

A Bulgarian study indicated similar results in candidemic patients on the basis of 38 *Candida* strains collected during a 5-year period (2007-2011) (17). According to the data in the review of Whaley et al. (9), *C. glabrata* was the main invasive NAC species in North America, Northern Europe, and some other regions, with the exception of Latin America, and it was a frequent etiological agent of vulvovaginal candidiasis and candiduria. In the present study, the predominant part of *C. glabrata* isolates (74.1%) was obtained from urine samples. Some authors also reported urine as a common site of *C. glabrata* isolation (15, 25), whereas the others detected *C. tropicalis* as a primary agent of candiduria (26, 27).

Our data concerning antifungal susceptibility showed decreased susceptibility to azoles in 73.13% of NAC. Furthermore, 26.87% of the isolates were resistant to all azoles. Savastano et al. (28) presented about 50% resistance to fluconazole among non-*albicans Candida*. As is well known, azole resistance is more common in NAC as compared to *C. albicans* (9). The results from Bulgarian trials confirmed these observations (16, 17, 29). In the current study, *C. glabrata* showed MIC_{50} and MIC_{90} of fluconazole 16 and > 128 μg/mL, until voriconazole MIC_{50} and MIC_{90} were considerably lower - 0.25 and 8 μg/mL. Similar MICs were observed in studying of 2379 *C. glabrata* isolates: fluconazole MIC_{50} and MIC_{90} - 10.6 and > 165 μg/mL, and voriconazole MIC_{50} - 0.63 μg/mL (20). The data for Bulgaria revealed fluconazole and voriconazole MIC_{90}s > 64 and 16 mg/L, re-

Table 4. Multidrug-Resistant Non-*albicans Candida* Isolates

Candida spp.	Sample	Antifungal Agents (MICs in μg/mL)								
		FCA	ITR	POS	VOR	ANF	CAS	MIF	FCY	APH
C. glabrata	Urine	16	> 4	2	1					2
C. tropicalis	Wound	> 128	> 4	> 8	> 8	> 8	4	> 8		
C. tropicalis	Bal	> 128	4	8	> 8				32	2
C. tropicalis	Wound	> 128	> 4	> 8	> 8				32	2
C. lipolitica	Urine	32	4	1	1	1	8	> 8		
C. famata	Urine	> 128	> 4	8	8					2

Abbreviation: MIC, minimum inhibitory concentrations; FCA, fluconazole; ITR, itraconazole; POS, posaconazole; VOR, voriconazole; ANF, anidulafungin; CAS, caspofungin; MIF, micafungin; FCY, 5-fluorocytosine; APH, amphotericin B.

spectively (16). These values were established in the testing of 21 *C. glabrata* collected between 2009 and 2013. According to a 4-year global evaluation, voriconazole demonstrated 1 to 2 logs larger action than fluconazole against all tested *Candida* species (12). Analogous results were obtained by other authors (15, 30).

Candida glabrata exhibits decreased intrinsic susceptibility to azoles and it is able to develop high-level resistance after azole exposure (10). The published data concerning this problem varied widely. Pfaller et al. (27) reported different rates of fluconazole resistance in *C. glabrata* among Latin American countries - from 5.9% in Brasilia to 36% in Venezuela. In a study, including 35 countries worldwide, about 81% of *C. glabrata* were susceptible or susceptible dose-dependent to fluconazole; however, there was an increasing rate of resistant strains from blood and upper respiratory tract samples (16). Surprisingly, we detected 3 *C. tropicalis* isolates with high-level resistance to all azoles. Kaur et al. (21) found that this species was more resistant to azoles than other *Candida* spp. – out of 37 *C. tropicalis* strains, 15 were resistant to fluconazole. While the increasing rate of fluconazole resistance in *C. tropicalis* is well documented (31), voriconazole resistance is extremely rare. Little is known about the mechanisms of azole resistance in this species (10). Recently, ERG11 overexpression was detected in *C. tropicalis* isolates from China and Korea, especially in fluconazole-resistant strains also resistant to itraconazole and voriconazole (32, 33).

Our data confirmed the intrinsic resistance of *C. krusei* to fluconazole, but MIC values (32 - 64 μg/mL) were considerably lower than those observed in Bulgaria (MIC_{90} > 256 mg/L) (16). This could be explained by the very small number of tested isolates. Furthermore, the MICs of posaconazole and voriconazole ranged between 0.0625 - 0.125 μg/mL, whereas the MICs of itraconazole were considerably higher (1 - 4 μg/mL). Azevedo et al. (15) found decreased susceptibility to fluconazole in about 70% of overall 54 *C. krusei* strains. The same workers determined fluconazole MIC_{90} of 146.76 μg/mL and voriconazole MIC_{90} of 0.37 μg/mL. According to Hazen et al. (20), *C. krusei* was the species with the highest voriconazole MIC_{50} - 1.5 μg/mL. Generally, the itraconazole resistance of NAC species varied in different reports- from 0 (34) to 33.3% (28). Iranian researchers (22) revealed itraconazole MICs: 0.125 - 4 μg/mL when testing 49 NAC strains.

Our results about echinocandins were in concordance with the conception of a low resistance rate among *Candida* species (35, 36). A possible reason for that is the limited use of echinocandins in our hospital. Pfaler et al. (36) established only 0.1% caspofungin resistance in 5,346 *Candida* isolates. In a multicenter Bulgarian study covering 106 *C. albicans* and 96 NAC strains, elevated MICs of anidulafungin were observed in 26 *C. parapsilosis* with MIC_{90} > 2 mg/L (16). In the present study, amphotericin B-resistance (5.97%) was less than azole resistance. These data collected are close to some reports (21) and are in contrast to others, presenting about 20% (17) - 25% (22) resistance to this drug in NAC species. Yüksekkaya et al. (23) did not find any amphotericin B-resistant strain in 56 *Candida* spp. We detected 6 (8.95%) multidrug-resistant isolates in our study. Taghipour et al. (22) also found four multiresistant strains among 49 NAC. Three of which were resistant to amphotericin B and itraconazole, and one to amphotericin B, itraconazole, and terbinafine.

5.1. Conclusions

About 70% of the tested 69 NAC isolates demonstrated decreased susceptibility to one or more azoles, and 18 of them were resistant to all azoles. These findings highlight the need for appropriate antifungal control programs in our institution.

Footnotes

Authors' Contribution: Study concept and design: Hristina Hitkova. Acquisition of data: Diana Georgieva, Preslava Hristova, Teodora Marinova-Bulgaranova. Analysis and interpretation of data: Hristina Hitkova, Diana Georgieva. Drafting the manuscript: Hristina Hitkova,

Vladimir Popov. Critical revision of the manuscript for important intellectual content: Biser Borisov.

Conflict of Interests: The authors declare that there is no conflict of interests in this study.

Ethical Approval: The study pattern, the informed consent form and diagnostic and assessment tools used were approved by the Research Ethics Committee at Medical University, Pleven. None of the procedures in the study posed a risk to the life and health of patients.

Funding/Support: This study was carried out with financial support of Medical University, Pleven through University Grants Commission (Project No. 16/2017).

Informed Consent: All patients signed written informed consent before the procedure started.

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